Single nucleotide polymorphisms in the CNTNAP2 gene in Brazilian patients with autistic spectrum disorder

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ABSTRACT.
The role of some genes and their single nucleotide polymorphisms (SNPs) as genetic contributors of complex diseases is still a topic of much investigation. Research on genes related to autism susceptibility has been somewhat challenging, but also promising. Common genomic variants of CNTNAP2 have been associated with autism, and a range of autistic phenotypes such as impaired language function, abnormal social behavior, intellectual deficiency, epilepsy, and schizophrenia have been associated with this gene. Earlier findings have suggested that SNPs in the CNTNAP2 gene may be used as genetic markers for predisposition to autism spectrum disorder (ASD). We analyzed the SNPs (rs7794745 and rs2710102) in the CNTNAP2 gene of 210 individuals with idiopathic ASD and 200 non-autistic individuals by polymerase chain reaction-restriction fragment length polymorphism. The
results revealed higher frequency distributions statistically significant ($P = 0.034$) of the homozygous SNP rs7794745 (presumed risk genotype) in ASD patients as compared with control subjects. The results also showed an association ($OR = 1.802$, $95\% CI = 1.054-3.083$, $P = 0.042$) between the same homozygous genotype and ASD, suggesting that it is a susceptibility factor for autism in this Brazilian population.

**Key words:** Autism; Caspr2; Predisposition; Genetic factors

**INTRODUCTION**

Autism spectrum disorder (ASD) are a set of heterogeneous neurodevelopmental conditions characterized by early-onset difficulties in the social-communication domain, repetitive behaviors, and restricted interests with varying levels of impairment throughout the development of the affected individual (Faras et al., 2010; Lauritsen, 2013). The prevalence of ASD is approximately 1:88; ASD occurs in all ethnic and social groups, with males being more predominately affected, with a ratio of 4:1 (Currenti, 2010; Chung et al., 2014; Lai et al., 2014).

Neither genetic factors nor the environmental components have been extensively characterized to allow for diagnosis and treatment of non-syndromic ASD. However, genome-wide association studies have amassed evidence suggesting the involvement of hundreds of genes and their associated genetic pathways (Loke et al., 2015). Research reveals that non-syndromic cases may have multiple genomic components, which suggests that investigations of underlying susceptibility genes in autism may be somewhat challenging (Sokolowsky et al., 2012; Poot, 2015).

The etiology of ASD is complex and can be determined in 5-25% of the cases (Devlin and Scherer, 2012; Buxbaum et al., 2014). Many proposed candidate genes act on the central nervous system, affecting neural development and synaptic activities in particular. This may lead to abnormalities in neurological development and to neurobehavioral disorders such as ASD (Perche et al., 2010; Ye et al., 2010). Genomic analysis revealed that single nucleotide polymorphisms (SNPs) in candidate genes can be associated with predisposition to these disorders and may provide possible explanations for the phenotypic variability in autism (Abrahams and Geschwind, 2008; Mefford et al., 2012).

The contactin-associated protein-like 2 (CNTNAP2) gene (MIM 604569) has been mapped onto chromosome 7q35-36, and is one of the largest genes in the human genome with 2.5 million bp. It encodes the transmembrane protein Caspr2, which is a member of the neurexin family that functions as cell adhesion molecules as well as receptors in the nervous system (Alarcón et al., 2008; Arking et al., 2008; Stein et al., 2011). This protein is localized at the juxtaparanodes of myelinated axons and may play a role in the differentiation and function of these structures. Based on genomic rearrangements and copy number variations, the CNTNAP2 gene has been implicated in predisposition to neurodevelopmental disorders such as autism. Exome sequencing, which covers <0.2% of the CNTNAP2 genomic DNA, has revealed numerous SNP in healthy individuals and in patients with neurodevelopmental disorders (Peñagarikano and Geschwind, 2012; Clemm von Hohenberg et al., 2013; Poot, 2015).

The SNPs rs7794745 (A/T) and rs2710102 (C/T) of the CNTNAP2 gene have been associated with childhood autism and with language impairment in non-autistic individuals (Arking et al., 2008; Scott-Van Zeeland et al., 2010; Whalley et al., 2011). Furthermore, recent structural
imaging studies showed that healthy individuals homozygous for the presumed risk allele rs7794745 (genotype TT) show significant changes in brain regions previously reported to be altered in ASD individuals (Alarcón et al., 2008; Tan et al., 2010). However, Jonsson et al. (2014) suggested that these SNPs do not have any major influence on autistic-like traits in Swedish children.

Current literature on the frequencies of these polymorphisms in different populations and their relations with autism is still unclear. Therefore, the biological role of the CNTNAP2 gene in ASD is still a study focus area. In this context, we present the preliminary results of an investigation on the SNPs rs7794745 and rs2710102 of the CNTNAP2 gene, which has been performed for the first time in Brazilians affected with ASD.

MATERIAL AND METHODS

Subjects

The study protocol was approved by the Ethics Research Committee of the School of Medicine in São José do Rio Preto. Written informed consent was obtained from all subjects or their guardians and from control individuals.

The study involved 410 individuals; 210 idiopathic ASD (174 males and 36 females, 13.4 ± 1.37 years old) patients were recruited into psychiatric clinics and specialized schools in São Paulo State, Brazil. ASD was diagnosed according to the DSM-5 (APA, 2013) following previously standardized criteria. All patients were evaluated for possible associated syndromes, as well as for potential environmental etiology and chromosomal abnormalities by conventional GTG-banding. Males were also tested for the FMR1 gene mutation by molecular tests. Individuals who had these alterations, were dysmorphic, or had hearing disorders were excluded from the study.

The control group included 200 healthy Brazilian individuals (137 males and 63 females; age, 34 ± 6.2 years) who had no psychiatric disorders and had no family history of such disorders for at least three generations or in the next generation. They were recruited among healthy blood donors from the Blood Center of São José do Rio Preto, São Paulo, Brazil.

Genotyping of SNPs

Blood was obtained from autistic probands and control individuals. Genomic DNA was extracted from whole peripheral blood samples by the Ficoll Paque method (Miller et al., 1988).

We selected two SNPs in the CNTNAP2 gene, rs7794745 and rs2710102, by in silico analysis. These SNPs were genotyped in all participants by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. Primer sequences and PCR-RFLP analysis are presented in Table 1. For the SNP rs2710102, PCR was carried out in a 20.0 µL reaction volume containing 9.7 µL ultrapure water, 2.5 µL reaction buffer, 0.8 µL MgCl2, 0.5 µL dimethylsulfoxide (100% DMSO), 1.0 µL 10 pM primer (sense and anti-sense), 2.0 µL 1.25 mM dNTP, 0.2 µL Taq polymerase, and 2.0 µL 50 ng/µL DNA. Amplification cycles were as follows: initial denaturation at 95° (5 min), followed by 35 cycles at 95°C (1 min), 59°C (30 s), 72°C (30 s), and extension at 72°C (5 min). An aliquot (7.0 µL) of the PCR product was completely digested at 37°C with 0.7 µL 5 U/µL restriction enzyme for 20 min. The digested DNA fragments were subjected to electrophoresis on a 3% agarose gel. The conditions used for PCR amplification of the rs7794745 SNP have been described by Li et al. (2010). An aliquot (7.0 µL) of the PCR product was completely digested at
65°C with 0.5 µL 5 U/µL of the restriction enzyme for 20 min. Electrophoresis was performed for digested DNA fragments using a 3% agarose gel.

**Statistical analysis**

A chi-square test ($\chi^2$) was applied to compare the overall frequencies of SNP genotypes between ASD individuals and the control group, as well as to verify whether genotype distributions were in Hardy-Weinberg equilibrium. The Fisher exact test was used to detect differences in the distribution of genotypes (recessive model) in both SNPs of the CNTNAP2 gene. Alpha error of 5% ($P < 0.05$) was considered acceptable. The analysis of the association between genotypes and predisposition to ASD was performed by calculating the odds ratio (OR) with a confidence interval (CI) of 95%.

**RESULTS**

The frequency of the presumed risk allele (T) of SNP rs7794745 was 0.43 in autistic patients; 43 individuals were homozygous for this polymorphism (TT), and 167 were A carriers (AA = 71 and AT = 96). There was no statistic difference in the frequency of genotypes for this group ($\chi^2 = 4.715, P = 0.095$, degree of freedom = 2). In the control group, we found a frequency of 0.38 for the risk allele; 25 individuals were homozygous for this polymorphism (TT), and 175 were A carriers (AA = 75 and AT = 100).

Comparative analysis of the distribution of the homozygous genotype for the presumed risk allele (TT) between individuals with ASD and the control group showed a statistically significant difference ($P = 0.034$) between the two groups. In addition, the results suggested an association between this specific genotype and ASD (OR =1.802; 95%CI = 1.054-3.083; $P = 0.042$) (Table 2).

**Table 2.** Allele and genotype frequencies of rs7794745 in ASD patients and healthy individuals.

<table>
<thead>
<tr>
<th>CNTNAP2 rs 7794745</th>
<th>ASD (N = 210)</th>
<th>Control (N = 200)</th>
<th>$P^*$</th>
<th>OR</th>
<th>$P^{**}$</th>
<th>95%CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genotypes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>71 (33.8%)</td>
<td>75 (37.5%)</td>
<td>0.470</td>
<td>0.852</td>
<td>0.488</td>
<td>0.568-1.268</td>
</tr>
<tr>
<td>AT</td>
<td>96 (45.7%)</td>
<td>100 (50.0%)</td>
<td>0.429</td>
<td>0.842</td>
<td>0.442</td>
<td>0.571-1.241</td>
</tr>
<tr>
<td>TT</td>
<td>43 (20.5%)</td>
<td>25 (12.4%)</td>
<td><strong>0.034</strong></td>
<td>1.802</td>
<td>0.042</td>
<td>1.054-3.083</td>
</tr>
<tr>
<td><strong>Alleles</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>238 (56.7%)</td>
<td>250 (62.5%)</td>
<td>0.102</td>
<td>0.785</td>
<td>0.103</td>
<td>0.593-1.038</td>
</tr>
<tr>
<td>T</td>
<td>182 (43.3%)</td>
<td>150 (37.5%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Calculated by the Fisher exact test. **Odds ratio. 95%CI = confidence interval.

The frequency of the presumed risk allele (C) of the CNTNAP2 rs2710102 SNP was 0.52. For this polymorphism, 50 individuals were homozygous (CC), and 160 were T carriers (CT: N

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**Table 1.** Primer sequences and PCR-RFLP analysis.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Primer sequence (5' - 3’)</th>
<th>Product (bp)</th>
<th>Restriction enzyme</th>
<th>Allele (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs 2710102</td>
<td>ATGGATGGACTGACCGATTG</td>
<td>198</td>
<td>Avai (Eco88I)</td>
<td>C (43/155)</td>
</tr>
<tr>
<td>rs 7794745</td>
<td>CAACATTGATCCCTACGCACT</td>
<td>311</td>
<td>Tsp509I</td>
<td>T (78/223)</td>
</tr>
</tbody>
</table>

PCR-RFLP = polymerase chain reaction-restriction fragment length polymorphism; SNP = single nucleotide polymorphism; bp = base pair.
Polymorphisms in the CNTNAP2 gene in a Brazilian population

In the control group, we found a frequency of 0.58 for the risk allele; 64 individuals were homozygous for this polymorphism (CC), and 136 were T carriers (TT = 32 and CT = 104). The frequencies of the C allele between the control and ASD groups were similar ($\chi^2 = 3.449; P = 0.176; DF = 2$), and the distribution of the risk genotype CC was also similar between the two groups ($P = 0.077$). This finding suggests that this allele on the rs2710102 SNP is not associated with ASD (OR = 0.664, 95%CI = 0.429-1.026, $P = 0.082$) (Table 3).

<table>
<thead>
<tr>
<th>CNTNAP2 rs 2710102</th>
<th>ASD (N = 210)</th>
<th>Control (N = 200)</th>
<th>P*</th>
<th>OR</th>
<th>P**</th>
<th>95%CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotypes</td>
<td>[N (%)]</td>
<td>[N (%)]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>41 (19.5%)</td>
<td>32 (16%)</td>
<td>0.369</td>
<td>1.274</td>
<td>0.422</td>
<td>0.765-2.119</td>
</tr>
<tr>
<td>CT</td>
<td>119 (56.7%)</td>
<td>104 (52%)</td>
<td>0.369</td>
<td>1.207</td>
<td>0.396</td>
<td>0.818-1.781</td>
</tr>
<tr>
<td>CC</td>
<td>50 (23.8%)</td>
<td>64 (32%)</td>
<td>0.077</td>
<td>0.684</td>
<td>0.982</td>
<td>0.429-1.026</td>
</tr>
<tr>
<td>Alleles</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>219 (52.1%)</td>
<td>232 (58%)</td>
<td>0.106</td>
<td>0.789</td>
<td>0.106</td>
<td>0.599-1.040</td>
</tr>
<tr>
<td>T</td>
<td>191 (47.8%)</td>
<td>168 (42%)</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

*Calculated by the Fisher exact test. **Odds ratio. 95%CI = confidence interval.

The rs7794745 and rs2710102 genotypes were found to be in Hardy-Weinberg equilibrium in the control group ($\chi^2 = 0.89, P = 0.345$ and $\chi^2 = 0.90, P = 0.341$). There was no evidence of linkage disequilibrium ($D' = 0.034$ and $r^2 = 0.001$) between the SNPs, which are 1 Mb from each other.

DISCUSSION

Converging evidence suggests that the CNTNAP2 gene is a strong candidate gene for predisposition to autism (Stein et al., 2011; Peñagarikano and Geschwind, 2012; Poot, 2015). Common genomic variants of CNTNAP2 have been associated with autism as well as related phenotypes such as impaired language function, abnormal social behavior, intellectual deficiency, epilepsy, and schizophrenia (Bakkaloglu et al., 2008; Friedman et al., 2008; Betancur, 2011; Miles, 2011; Angelidou et al., 2012).

In this study, we found that the frequency of the T allele of the CNTNAP2 SNP rs779475 in autistic patients (0.43) was similar to the frequency of 0.47 described in the database dbSNP polymorphisms/MAF (MinorAlleleCount) of the National Center for Biotechnology Information (NCBI). We found significantly higher frequency of the TT genotype of SNP rs7794745 in individuals diagnosed with ASD than in healthy subjects. The presence of the risk allele T in its homozygous form has been associated with altered activation of brain areas responsible for language in non-autistic individuals (Whalley et al., 2011). Language deficits are also core features of ASD. Genetic variations and abnormal gene expression of CNTNAP2 may increase the risks for specific language impairments by altering brain function during linguistic processing, which could result in manifestations such as found in individuals with ASD (Rodenas-Cuadrado et al., 2014). A positive association between the TT genotype and predisposition for autism has been observed by other researchers (Arking et al., 2008; Tan et al., 2010; Poot, 2015). Therefore, based on the results of our study, we suggest that a common variant of CNTNAP2 (genotype TT for rs7794745) can contribute to susceptibility to autism, which is in agreement with previous findings.

Our results showed that the frequency of the C allele (0.52) of the CNTNAP2 SNP rs2710102 in autism was also similar to the frequency described in SNP/MAF, which is 0.60 (NCBI).
Although some studies have proposed associations between the polymorphism rs2710102 and predisposition to autism (Alarcón et al., 2008; Scott-Van Zeeland et al., 2010), we did not find a significant difference between the frequencies of the CC risk genotype in the autistic and control groups. Similarly, other groups have also reported no association between this genotype and ASD in different populations (Whalley et al., 2011; Sampath et al., 2013). It was concluded that data on association between common variants of the CNTNAP2 gene and ASD have high variability. For example, Peñagarikano and Geschwind (2012) presented a review of more than 10 studies on variants of the CNTNAP2 gene, suggesting a possible association between these genotypes with ASD and other psychiatric disorders. In contrast, Toma et al. (2013) performed a case-control association study involving 322 Spanish autistic patients and 524 controls, which suggested that rs2710102 and rs7794745 are unlikely to contribute to susceptibility to autism.

Ethnic characteristics of populations may result in distinct genetic susceptibility for the same diseases. As a result of five centuries of cross-breeding between Amerindians, Europeans, and Africans, the Brazilian population displays very high levels of genomic diversity, with a predominant European ancestry in different regions (Pena et al., 2011; Huguet et al., 2013) as the one studied here (São Paulo State). Thus, the genetic heterogeneity and admixture of Brazilians may have important implications in studies of genetic markers, including SNPs (Lee et al., 2013). Analysis of susceptibilities to complex diseases such as ASD may require different genetic markers for different populations, which can be important in the practice of clinical genetics and the etiology of these diseases.

The contribution of genes and their SNPs to complex diseases is still an open question. Moreover, the frequency of individual alleles is influenced by the sizes in the study groups (Murdoch and State, 2013). Therefore, future studies should aim to include clinical details of patient samples, which may clarify the significance of these SNPs for the risk of autism.

Therefore, the TT genotype (rs7794745) of the CNTNAP2 gene may be associated with predisposition to autism in the Brazilian population. We suggest that it can at least increase the risk for ASD, considering that SNPs in complex diseases generally have combined effects.

Conflicts of interest

The authors declare no conflict of interest.

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